The Replication of Human Immunodeficiency Virus Type 1 in Macrophages Is Enhanced after Phagocytosis of Apoptotic Cells

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Clearance of apoptotic cells increases macrophage secretion of antiinflammatory mediators and might modulate viral replication in human immunodeficiency virus (HIV) type 1–infected macrophages. To study this, primary macrophages were infected with HIV-1 and exposed to apoptotic cells. It was found that phagocytosis of apoptotic cells potently enhanced HIV-1 growth. The peptide Arg-Gly-Asp-Ser, which binds to integrin receptors, inhibited the uptake of apoptotic cells and the subsequent enhancement of HIV-1 replication. Viral replication was preceded by increased secretion of transforming growth factor (TGF)–β1 and partially reverted by anti–TGF-β1 antibodies. Moreover, anti–TGF-β1 antibodies inhibited HIV-1 replication in macrophages not exposed to apoptotic cells. A positive correlation was observed between TGF-β1 production and HIV-1 growth, and the addition of TGF-β1 amplified HIV-1 replication in macrophages from low TGF-β1 producers. The findings suggest that TGF-β1 favors HIV-1 replication in macrophages and that the clearance of apoptotic cells by HIV-1–infected macrophages contributes to persistent viremia in patients infected with HIV-1.

Human immunodeficiency virus (HIV) type 1, the etiological agent of AIDS, induces apoptosis in HIV-1–infected and uninfected CD4+ T cells, which probably contributes to the progressive loss of CD4+ T lymphocytes in infected individuals [1–3]. Accordingly, the number of apoptotic CD4+ cells is significantly augmented in lymphoid tissues of individuals infected with HIV-1, compared with the number in uninfected controls [4]. Macrophages are characteristically resistant to virus-mediated killing, functioning as an HIV-1 reservoir [5], and apoptotic cells are in close proximity to a large number of HIV-1–infected macrophages in lymphoid tissues [5, 6]. Apoptotic cells are promptly phagocytized by macrophages, which are equipped with a number of receptors that recognize and bind to molecules expressed by apoptotic cells [7]. The clearance of apoptotic bodies by macrophages induces the secretion of antiinflammatory cytokines [8, 9] such as transforming growth factor (TGF)–β1 [9], although its role in HIV-1 replication is still a matter of debate [10–12]. Recently published data showed that TGF-β1 released by apoptotic T cells suppresses macrophage activation [13], and other studies demonstrated that the uptake of apoptotic cells by macrophages may trigger suppressive properties in these phagocytes. In fact, exposure to apoptotic cells resulted in impairment of macrophage-mediated tumor death [14] and exacerbated parasite growth in *Trypanosoma cruzi*–infected murine macrophages, a phenomenon in which TGF-β1 played a major role [15]. Since phagocytosis of apoptotic cells modifies macrophage secretion of a variety of cytokines that can influence HIV-1 replication [16], including TGF-β1 [9], we investigated whether this event might modulate the growth of HIV-1. The continual removal of apoptotic cells by infected macrophages could lead to an exacerbated viral replication in this HIV-1 reservoir, which might contribute to raising the virus load in individuals infected with HIV-1.

Materials and Methods

Virus, reagents, and cells. All assays of macrophage infection were done with the monocytotropic, CCR5-dependent isolate HIV-1Ba-L (donated by Michael A. Norcross, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD), which was expanded in phytohemagglutinin-activated peripheral blood mononuclear cells (PBMC). The peptides Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES) were purchased from Sigma Chemical. Recombinant human cytokine TGF-

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β1 (rhTGF-β1) and purified chicken anti–human TGF-β1 IgY antibodies were from R&D Systems. The isotype control (chicken IgY; donated by George A. DosReis, Instituto de Biofísica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil) was prepared as described elsewhere [17]. Monocyte-derived macrophages from healthy donors were isolated by plastic adherence of PBMC previously obtained by density gradient centrifugation (Histopaque; Sigma).

For HIV-1 infection assays, 4×10^6 PBMC were plated in 24-well plates in RPMI medium without serum for 1 h in 5% CO_2 at 37°C. Non-adherent cells were washed out, and adherent cells were maintained with Dulbecco's modified Eagle medium (DMEM) with 10% human serum (Sigma) for 7 days for differentiation in macrophages. Macrophage purity was >90%, as determined by flow cytometry (FACScan; Becton Dickinson) analysis using anti-CD3 (PharMingen) and anti-CD14 (PharMingen) monoclonal antibodies. Apoptotic cells were prepared from the CD4$^+$ T cell line Jurkat by culturing 10^6 cells/mL in RPMI medium supplemented with 1% fetal calf serum for 18–20 h at 37°C in 5% CO_2. Apoptotic cells were prepared from PBMC by heating 10^6 cells/mL at 44°C in RPMI medium supplemented with 10% fetal calf serum for 1 h and then culturing them at 37°C in 5% CO_2 for 4–6 h. These treatments induced apoptosis in 80% of the cells, as determined by DNA fragmentation and annexin V staining (data not shown). Fixed cells were obtained by treating Jurkat cells or PBMC with 1% paraformaldehyde for 30 min at room temperature, followed by extensive washing with RPMI medium.

Phagocytosis of apoptotic cells. To measure the uptake of apoptotic cells, 10^6 PBMC were seeded in 8-well Permanox chamber slides (Nalge Nunc), and macrophage differentiation was performed as above. Next, apoptotic cells were added to macrophages at a ratio of 10:1 for 1 h. Nonphagocytized cells were washed out, and macrophages were fixed and stained with hematoxylin-eosin. Phagocytic indices (PIs) were determined as the percentage of macrophages that had phagocytized apoptotic cells, multiplied by the average number of ingested cells per macrophage, as described elsewhere [9], and they are presented as the percentage of the PI obtained with control uninfected macrophages. Macrophages were also exposed to fixed PBMC or fixed Jurkat cells, under the same conditions as above.

Macrophage infection and exposure to apoptotic cells. Macrophages were infected using 10–20 ng/mL HIV-1 p24 antigen. After incubation overnight, excess virus was washed out, and apoptotic or fixed cells were added to infected macrophages at a 10:1 ratio. After 2 h at 37°C (5% CO_2) in DMEM without serum, unphagocytized apoptotic cells were removed, and monolayers were replenished with fresh medium supplemented with 10% human serum. Cultures were maintained for 3 weeks, and half of the culture medium was renewed each 7 days. Viral replication was measured in culture supernatants by use of a p24 antigen ELISA capture assay (Cellular Products). TGF-β1 levels in culture supernatants were measured by ELISA (Genzyme Diagnostics) after acidic activation, according to the manufacturer's instructions. To test the participation of integrin receptors, we added RGDS or RGES peptides (2 mM) to macrophages 10 min before exposure to apoptotic cells. To neutralize the activity of TGF-β1, we added anti–TGF-β1 (or the isotype IgY) antibodies to cultures at 10 μg/mL immediately after the removal of apoptotic cells. In some experiments, exogenous rhTGF-β1, at final concentrations of 1, 3, and 10 ng/mL, was added immediately after cell infection to HIV-1–infected macrophages. For these tests, macrophages were selected according to their levels of spontaneous production of TGF-β1 and classified as low (<120 pg/mL) or high (>800 pg/mL) TGF-β1 producers.

Statistical analysis. Student's t and Spearman's r correlation tests were used to determine statistical significance, which was defined as $P < .05$.

Results

We initially evaluated the PI of HIV-1–exposed or –unexposed macrophages. After feeding the cells with apoptotic Jurkat cells, we found that the PI for HIV-1–infected macrophages was equivalent to 99% of that for uninfected macrophages ($n = 4$). The PI for autologous apoptotic PBMC in HIV-1–infected macrophages was 137%, relative to the PI for uninfected cells ($n = 3$). These PIs confirm that the phagocytic characteristics of the HIV-1–exposed macrophage population are not altered, as demonstrated elsewhere [18, 19]. The PI for autologous fixed PBMC in HIV-1–infected macrophages was similar (113%) to the PI for the uninfected macrophages, but it reached only 47% and 56% ($n = 3$) of the PI for apoptotic PBMC in the infected and uninfected macrophage populations, respectively, suggesting that the uptake of fixed cells is less efficient than the phagocytosis of apoptotic cells.

The recognition and uptake of apoptotic bodies by macrophages involve many receptors, including the $\alpha_V \beta_3$ integrin (CD51/61) [20–23], which displays high-affinity binding to proteins or peptides containing an Arg-Gly-Asp (RGD) sequence [24]. As reported elsewhere [22], treatment of macrophages with RGDS (2 mM) led to a 50% reduction in the uptake of apoptotic cells, whereas the control peptide RGES did not inhibit the phagocytic process (98%), compared with that for macrophages in the presence of medium only ($n = 3$; $P = .03$).

The exposure of infected macrophages to apoptotic Jurkat cells potently amplified HIV-1 replication (figure 1). Phagocytosis of apoptotic cells induced a 5–13-fold increase in HIV-1 replication 7 days after infection, and it induced a 5–26-fold increase after 14 days, compared with viral growth in macrophages not exposed to apoptotic cells ($n = 3$; $P = .004$). The amplified viral replication persisted during the subsequent week, although it was less pronounced (2–6-fold over HIV-1 production in the absence of apoptotic cells). The same enhancing effect was obtained with heterologous apoptotic PBMC, with increments in HIV-1 replication of 1.5–8-fold, 3.5–5-fold, and 3–8-fold after 1, 2, and 3 weeks of cell infection, respectively, compared with viral growth in the absence of apoptotic cells ($n = 3$; $P = .03$, after 14 days of infection). Of note, exposure of infected macrophages to autologous apoptotic, but not to autologous paraformaldehyde-fixed PBMC, resulted in an equivalent amplification phenomenon (mean $\pm$ SEM, 15.5 ± 8.6–fold vs. 1.6 ± 0.6–fold the control after 3 weeks; $n = 4$; $P = .19$).
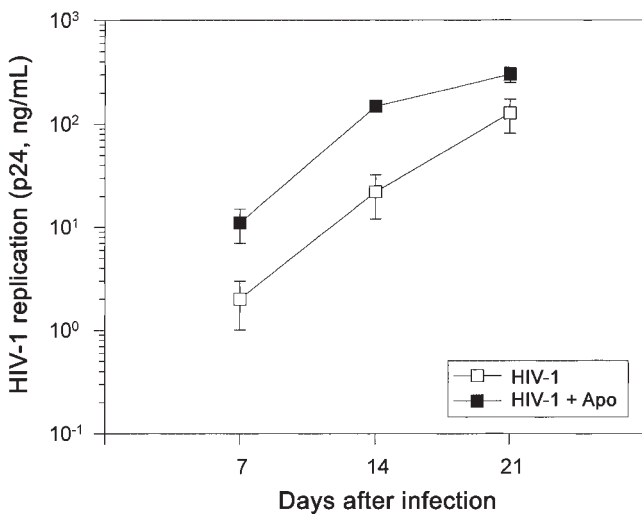
Figure 1. Effect of phagocytosis of apoptotic cells on human immunodeficiency virus (HIV) type 1 replication. Macrophages were infected with HIV-1$_{Ba-L}$ and provided with fresh medium (HIV-1) or exposed to apoptotic Jurkat cells (HIV-1 + Apo) for 2 h. Cultures were replenished with half a volume of fresh medium weekly, and viral replication was measured in the culture supernatants by an HIV p24 ELISA at different time points, as indicated. Data represent the means ± SEM of 3 independent experiments. $P = .004$ after 14 days of culture, HIV-1 + Apo vs. HIV-1.

Control tests with fixed Jurkat cells also did not induce amplification of HIV-1 growth (1.7 ± 0.6–fold the control; $n = 4$). Phagocytosis of apoptotic cells resulted in an exacerbated HIV-1 replication in all individuals tested at all time points. However, a statistically significant increase was observed only after 14 days of infection. For example, in additional experiments ($n = 11$), the viral growth in macrophages exposed to apoptotic Jurkat cells was equivalent to 11.6 ± 3.2–fold of HIV-1 replication in infected macrophages not exposed to apoptotic cells ($P = .008$).

Once we demonstrated that the RGDS peptide inhibited the uptake of apoptotic cells, we investigated whether it could revert the enhancement of HIV-1 replication driven by ingestion of apoptotic cells. We found that pretreatment of infected macrophages with RGDS, but not with RGES, almost totally ablated ($93\% \pm 1.5\%$; $n = 3$; $P = .19$) the augmentation of HIV-1 growth (figure 2). The addition of RGDS and RGES to HIV-1–infected macrophages in the absence of apoptotic cells did not inhibit the spontaneous viral replication (data not shown). These results imply the participation of an integrin receptor in the amplified viral replication driven by uptake of apoptotic cells.

It has been reported elsewhere that phagocytosis of apoptotic cells increases TGF-β1 production by macrophages [9]. Thus, we studied whether this cytokine participated in the up-regulation of HIV-1 infection. We found that exposure to apoptotic Jurkat cells caused an ∼2-fold increase in TGF-β1 secretion in

uninfected (2621 ± 981 vs. 1473 ± 751; $n = 4$; $P = .28$) and HIV-1–infected macrophages (1368 ± 406 vs. 762 ± 444; $n = 4$; $P = .12$) (figure 3). These data did not reach statistical significance because of strong donor-to-donor variation in TGF-β1 production, as observed elsewhere (J.V.W. and M.B.N., unpublished data). Similar results were detected with apoptotic PBMC (data not shown). Furthermore, inhibition of TGF-β1 activity by adding neutralizing antibodies partially blocked ($58\% \pm 4\%$; $n = 3$; $P = .14$) the exacerbation of viral growth (figure 4), which implies that TGF-β1 participates in this phenomenon. Moreover, anti–TGF-β1 antibodies significantly reduced ($67\% \pm 5\%$; $n = 3$; $P = .007$) the spontaneous replication of HIV-1 in infected macrophages, indicating that endogenously produced TGF-β1 favors HIV-1 replication even in the absence of any extra stimulus (figure 4). Accordingly, we observed a strong positive correlation between TGF-β1 production and HIV-1 growth in macrophages after 7 (Spearman's $r = 0.96$; $P = .002$) and 14 (Spearman's $r = 0.66$; $P = .026$) days of infection in cells of 10 individuals used throughout this study. We further analyzed the role of this cytokine on HIV-1 replication by adding rhTGF-β1 to HIV-1–infected primary macrophages. The addition of exogenous rhTGF-β1 boosted HIV-1 replication in cells from donors who produce low levels of TGF-β1, whereas no effect was observed in high TGF-β1 producers (figure 5).
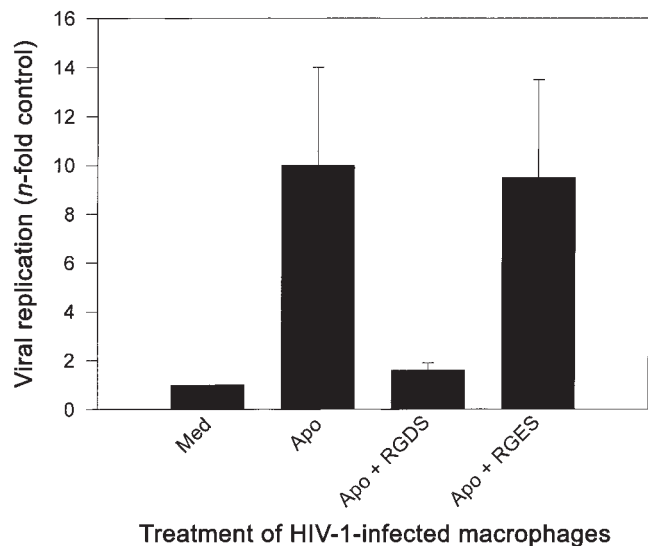

Figure 2. Enhancement of human immunodeficiency virus (HIV) type 1 replication is neutralized by Arg-Gly-Asp-Ser (RGDS). Macrophages were infected with HIV-1$_{Ba-L}$, replenished with fresh medium and left untreated (Med), exposed to apoptotic Jurkat cells (Apo), or treated with the peptides RGDS (Apo + RGDS) or RGES (Arg-Gly-Glu-Ser; Apo + RGES) 10 min before addition of apoptotic cells. HIV-1 replication was measured in culture supernatants by an HIV p24 ELISA 14 days after infection. Data represent the means ± SEM of 3 independent experiments and are shown as virus production over control (Med), arbitrarily defined as 1. Peptides alone did not inhibit the spontaneous viral replication.
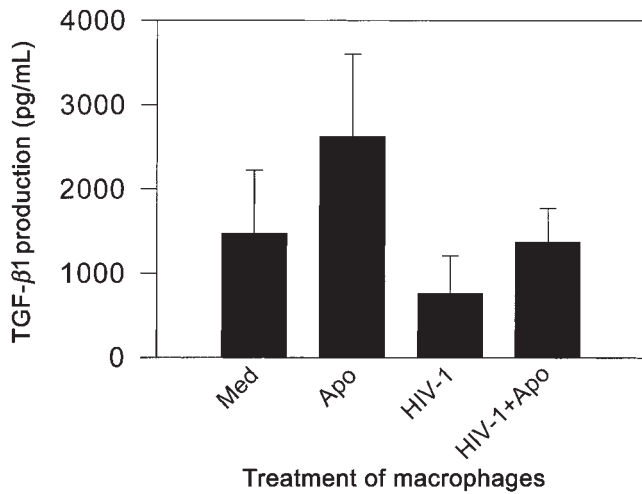
Figure 3. Production of transforming growth factor (TGF)–β1 by uninfected or human immunodeficiency virus (HIV) type 1–infected macrophages after phagocytosis of apoptotic cells. Uninfected and HIV-1$_{Ba-L}$–infected macrophages were cultured with medium only (Med or HIV-1, respectively) or exposed to apoptotic Jurkat cells (Apo or HIV-1 + Apo, respectively) for 2 h. Unphagocytized cells were removed, fresh medium was added, and levels of TGF-β1 were measured in acid-treated culture supernatants 7 days later, by use of an ELISA kit. Data represent means ± SEM for 4 different donors.

Discussion

Macrophages play a central role in the pathogenesis of HIV-1 infection, functioning as an HIV-1 reservoir through their ability to resist HIV-1–mediated cytopathicity and continuously replicate the virus [5, 6]. Production and release of infective virions by means of macrophages are probably influenced by a network of factors acting in concert where macrophages reside and exert physiologic activities. In this regard, we addressed whether the uptake of apoptotic cells might modulate viral replication in HIV-1–infected macrophages and found that this regular phagocytic activity consistently up-regulated HIV-1 replication in these cells, a phenomenon involving an integrin receptor and the cytokine TGF-β1.

We initially observed that addition of apoptotic Jurkat cells to HIV-1–infected macrophages powerfully augmented viral replication (figure 1). We also performed similar experiments using apoptotic PBMC, which also resulted in vigorous and consistent enhancement of HIV-1 growth. In addition, like Jurkat cells, apoptotic PBMC induced elevated secretion of TGF-β1 by means of uninfected and infected macrophages (data not shown).

Increases of virus load and viral persistence in HIV-1–infected individuals have been associated with a variety of conditions, such as coinfections [25–27] and immune activation [28, 29]. It has been proposed that the permissiveness of macrophages to HIV-1 infection is enhanced in such circumstances [27], resulting in a higher number of HIV-1–infected and virus-producing macrophages. Here, we describe that acutely HIV-1–infected

macrophages are stimulated to produce and release an increased amount of virions following a physiologic event, namely the phagocytosis of apoptotic cells. Clearance of apoptotic cells is not a silent process, because it can reverberate in macrophage physiology [8, 9, 14, 15], and our findings may denote a macrophage inability to control its own HIV-1 infection, secondary to alterations induced by the uptake of apoptotic cells. Thus, the prevailing process of clearance of constitutive or HIV-1–induced apoptotic lymphocytes could be a contribution to persistence of virus load in the macrophage reservoir in vivo.

We demonstrated that the uptake of apoptotic cells by HIV-1–infected macrophages, through an integrin receptor, up-regulates viral replication, suggesting that signaling originated from this family of receptors may stimulate HIV-1 production in infected macrophages. In fact, it has been shown that engagement of integrin receptors on HIV-1–infected macrophages influences viral growth. For instance, up-regulation of HIV-1 replication was observed during macrophage-endothelial cell contact, requiring the engagement of the leukocyte function–associated antigen–1 [30], of which surface expression is significantly increased in HIV-1–infected macrophages [31]. Furthermore, attachment of HIV-1–infected monocytes to laminin induced a significant increase in viral replication [32]. In our model, the addition of RGDS resulted in 50% inhibition of apoptotic cells uptake but led to a 93% block of the increment of HIV-1 repli-
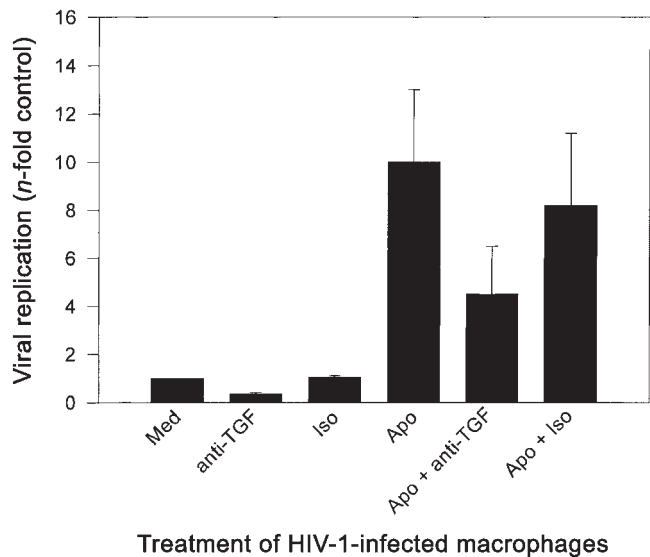

Figure 4. Enhancement of human immunodeficiency virus (HIV) type 1–replication is partially inhibited by anti–transforming growth factor (TGF)–β1 antibodies. Macrophages were infected with HIV-1$_{Ba-L}$ and left untreated (Med) or treated with anti-TGF-β1 antibodies (anti-TGF) or isotype control (Iso) in the absence or presence (Apo, Apo + anti-TGF, Apo + Iso) of apoptotic Jurkat cells. HIV-1 replication was measured in the culture supernatants by an HIV p24 ELISA 14 days after infection. Data represent means ± SEM of 3 independent experiments and are shown as virus production over control (Med), arbitrarily defined as 1.
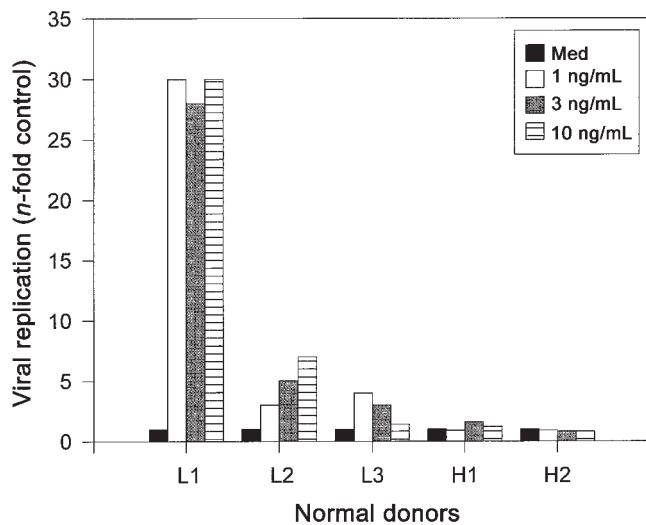
Figure 5. Effect of the addition of exogenous recombinant human transforming growth factor (rhTGF)–β1 on human immunodeficiency virus (HIV) type 1 replication in macrophages. Macrophages were infected with HIV-1$_{Ba-L}$, and rhTGF-β1 was added to cultures as indicated. HIV-1 replication was measured by p24 ELISA after 14 days of infection. Viral replication is shown as times over control (Med), arbitrarily defined as 1. Donor cells were from low (L1 = 120 pg/mL; L2 < 40 pg/mL; L3 = 45 pg/mL) or high (H1 = 830 pg/mL; H2 = 1200 pg/mL) TGF-β1 producers, as defined in Materials and Methods.

cation (figure 2), which showed that the engagement of RGD-binding receptors is crucial in this latter phenomenon. Because the $\alpha_V\beta_3$ vitronectin receptor recognizes apoptotic lymphocytes [33] and has recently been shown to play a critical role in the exacerbated parasite multiplication in murine macrophages infected with *T. cruzi* and exposed to apoptotic cells [15], it is most probable that this same receptor mediates the amplification of HIV-1 replication observed in this study.

Interaction of human macrophages with apoptotic cells inhibits the production of cytokines that activate HIV-1 replication [9], such as interleukin-1β, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor–α (for a review, see [16]), and stimulates secretion of TGF-β1 [9]. Moreover, TGF-β1 also participates in the increased *T. cruzi* growth in murine macrophages after engulfment of apoptotic cells [15]. Therefore, a possible participation of this cytokine in the up-regulation of HIV-1 replication in our system was evaluated, and we found that TGF-β1 mediated, at least partially, the enhancement of viral growth. The role of TGF-β1 on HIV-1 replication in vitro is controversial, however. Some authors found that TGF-β1 up-regulates viral replication in acutely infected primary macrophages [10], but others showed a suppressive effect, using either primary macrophages or the chronically HIV-1–infected promonocytic cell line U1 [11]. Recently, it was shown that TGF-β1 might limit virus production in peripheral blood lymphocytes resulting from TGF-β1–induced CD4$^+$ cell

death [12]. In our study, we provide strong evidence that TGF-β1 is an up-regulator of HIV-1 replication in primary macrophages, independently of the presence of apoptotic cells. The bimodal effect of exogenously added TGF-β1 on HIV-1 replication in cells of low and high TGF-β1 producers (figure 5) might explain the conflicting findings between different research groups [10, 11]. Thus, depending on previous priming by endogenous TGF-β1, the addition of this cytokine may induce supraphysiological stimulation, leading to decreased viral replication, whereas unprimed cells display enhanced viral replication in response to exogenously added TGF-β1. As anti–TGF-β1 antibodies did not completely revert the HIV-1 growth driven by uptake of apoptotic cells, other factors modulated by phagocytosis of apoptotic bodies [9] might be involved in this process.

Similar to what has been shown for parasitic diseases [15, 34], our findings suggest that TGF-β1 deactivates the ability of macrophages to control viral growth, allowing HIV-1 to replicate freely. The augmentation of the HIV-1 production may originate either from an increased number of infected macrophages, a higher multiplication of virions in individual cells, or both. Of interest, the autocrine effect on viral replication mediated by HIV-1 Tat protein may be analogous to the mechanism described here, because the Tat protein interacts with the vitronectin receptor [24, 35], induces overproduction of TGF-β1 [36], and exacerbates HIV-1 replication in infected cells [37]. In conclusion, we report a novel mechanism inducing pulses of HIV-1 production, through which HIV-1–infected macrophages might support the chronic persistence of viremia in HIV-1–infected patients. We suggest that pharmacologic interference with TGF-β1 production, biological activity, or its downstream signalization pathway might be considered as future targets in AIDS therapy.

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